

BIOMARKERS OF EXPOSURE AND EFFECT IN A LACERTID LIZARD
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Abstract—In Europe, reptiles have been recently included in environmental risk-assessment processes for registration of plant-protection products. However, data on toxicity effects of most compounds are lacking. Chlorpyrifos is the most commonly used organophosphorus insecticide worldwide. In the present study, the authors exposed a lacertid lizard, *Podarcis bocagei*, to sublethal concentrations of chlorpyrifos. Individuals were exposed through spiked food for a period of 20 d (low dose 0.12 mg/kg/d, high dose 1.57 mg/kg/d). After exposure, various biomarkers of exposure and effect were evaluated, including the activities of glutathione S-transferase and enzymes involved in the glutathione redox cycle, glutathione concentrations, activities of esterases, liver and testes histopathologies, as well as locomotory and predatory behavior. The results indicate that sublethal, subchronic exposure to chlorpyrifos can affect *P. bocagei* in a dose-dependent manner. Adverse effects occurred at both the subindividual and individual levels, including inhibition of carboxylesterases and cholinesterases (ChEs), liver histopathological changes, and altered predatory behaviors. Animals exposed to chlorpyrifos took more time to capture and subdue prey items. The results suggest a link between effects at subindividual levels of organization with those observed at the whole individual level after exposure to environmentally realistic dosages of chlorpyrifos. Environ. Toxicol. Chem. 2012;31:2345–2353. © 2012 SETAC

Keywords—Organophosphate Feeding study Reptile Ecological risk assessment Terrestrial ecotoxicology

INTRODUCTION

Globally, chlorpyrifos (*O,O*-diethyl-*O*-3,5,6-trichloro-2-pyridyl-phosphorothioate) is one of the most commonly used organophosphorus (OP) insecticides in agricultural areas. Chlorpyrifos tops the list of the 10 most used insecticides in the European Union, with an annual consumption of 1,226 tons of active substance [1]. As a member of the OP family, chlorpyrifos inhibits the hydrolytic activity of cholinesterases (ChEs) [2], and exposure to this pesticide is commonly evaluated by determination of inhibition of acetylcholinesterase (AChE; Enzyme Commission [EC] 3.1.1.7) in nervous tissue. Nevertheless, adverse effects of chlorpyrifos are not exclusively limited to neurotoxicity; and it has been shown to induce oxidative stress [3] and genotoxicity [4] and to affect reproductive parameters [5] and locomotor performance [6] in different vertebrate groups. The effects of chlorpyrifos have been extensively studied in different nontarget organisms in the aquatic environment [7,8] and in laboratory bioassays using rats as an animal model as well as in mammalian organs and tissues [9]. However, the detrimental effects from chlorpyrifos exposure in terrestrial ecosystems are less understood.

Reptiles, like other terrestrial vertebrates, respond to OP pesticides through a demonstrable inhibition of both brain and plasma ChE activities (S.R. Schmidt, 2003, Master's thesis, Texas Tech University, Lubbock, TX, USA). Several studies

have examined the toxic effects of OP insecticides in reptiles, measuring ChE activities [10,11] as well as other physiological and behavioral parameters [12,13]. Reptiles have seldom been considered in risk-assessment processes of pesticides, and toxicity data from surrogate species such as birds are commonly used to represent adverse effects in reptiles [14]. These extrapolations, while not always valid, assume that birds are equally or more sensitive to contaminants and that contaminant exposure is higher in birds [14]. Under the new European Commission Regulation 1107/2009 concerning the placement of plant-protection products on the European Union market, reptiles would be considered as nontarget organisms to be included in the risk-assessment framework for plant-protection product authorization. However, their inclusion depends on the availability of exposure and toxicity data, which are currently lacking.

The mode of action of environmental contaminants is a focus of predictive ecotoxicology [15]. Conceptual frameworks that try to link effects at the level of molecular or biochemical systems with adverse effects observed at the whole individual level are increasingly common in predictive risk-assessment processes [16,17]. An understanding of the mechanisms of toxic action in nontarget organisms is helpful to establish a relationship between the interaction of the pesticide at the target site and the subsequent adverse effects at the whole individual level. Available data from the scientific literature have enabled this mechanistic approach with regard to several classes of environmental contaminants, such as narcotic chemicals, estrogen receptor agonists (e.g., nonylphenol), aryl hydrocarbon receptor agonists (e.g., 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, planar polychlorinated biphenyls), and vitellogenesis disruptors

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(e.g., 17 β -trenbolone, prochloraz) [16]. Anticholinesterase pesticides are applicable candidates for this approach because of the large volume of information on their mechanism of action as well as on their potential adverse effects on organisms.

The aim of the present study was to examine the sublethal effects of chlorpyrifos at multiple levels of biological organization in the lizard *Podarcis bocagei* (Seoane, 1885). The attempt was to establish a direct link between biochemical processes of chlorpyrifos toxicity and detrimental effects at the individual level using a conceptual approach named "adverse outcome pathways" [16,17]. In Mediterranean environments, reptiles constitute a large component of agricultural ecosystems [18]. Moreover, lacertid lizards have been recently proposed as potential model species for reptile ecotoxicology in Europe [19]. *Podarcis* spp., the common wall lizards, are ubiquitous through most of central and southern Europe and have been used in a variety of studies to assess exposure to different contaminants such as metals [20], pesticides [21], and polycyclic aromatic hydrocarbons [22].

MATERIALS AND METHODS

Animal maintenance

Adult male *P. bocagei* were collected in the coastal dune system of Mindelo, Vila do Conde (northwestern coast of Portugal), in April 2010. Animals were housed individually in glass terrariums (40 \times 20 \times 25 cm) in a climate-controlled room (22 \pm 1°C). Terrariums contained a terra-cotta vase (diameter, 16 cm) that provided a refuge and a basking location and a 25-W incandescent lamp that created a thermal gradient (25–35°C, 8 h/d). Lighting was provided by natural sunlight, fluorescent lighting (2 \times 40 W), and a high-pressure sodium lamp (400 W, 7,000–12,000 Lx) for 12 h/d. Water was provided in shallow dishes and renewed every 2 d. Animals were acclimatized for 10 d before the start of the experiment and fed daily live mealworms (*Tenebrio molitor*).

Pesticide exposure conditions and tissue preparation

A total of 36 animals were randomly assigned to three experimental groups: control, low, and high dose. Nominal concentrations of the treatment solutions were 96 mg/L (low dose) and 960 mg/L (high dose). These chlorpyrifos concentrations were based on the application rate of the product in agricultural areas (150/200 ml/h) and a previous study that reported chlorpyrifos residue concentrations in mealworms (1 d, 0.53 mg/kg; 5 d, 5 mg/kg; and 10 d, 6.78 mg/kg [23]). Solutions were prepared daily by diluting in distilled water the commercial product Ciclone[®] 48 EC (480 g/L chlorpyrifos with xylene and other nondefined surfactants; Sapec Agro), covering, and agitating for 30 min before administering to mealworms using microliter syringes.

Lizards were fed every 2 d with two live mealworms (2–3 cm length), previously injected with 5 μ l of the corresponding chlorpyrifos solution (low and high dose) or distilled water (control group) over a period of 20 d. At the end of the exposure period, six individuals of each treatment were weighed, anesthetized by cooling on ice, killed by decapitation, and dissected. Animal handling complied with Portuguese animal ethics guidelines as stipulated by *Direcção Geral de Veterinária* and *Instituto da Conservação da Natureza e Biodiversidade*. Blood was collected with a pipette from the exposed trunk and centrifuged (800 rpm for 10 min at 4°C) to separate serum, which was frozen in liquid nitrogen. Liver and the left testis were weighed. Intestine, a portion of liver (the two larger lobes),

brain, and right testis were frozen in liquid nitrogen and stored at –80°C. Tissue samples were later thawed on ice and homogenized in 0.1 M Tris-HCl, 0.25 M sucrose, and 1 mM ethylenediaminetetraacetic acid (EDTA; pH 7.6) using a glass-Teflon Potter-Elvehjem homogenizer. Homogenates were centrifuged (10,000 g for 20 min at 4°C). A 100- μ l aliquot of the supernatant (postmitochondrial fraction) was mixed with 100 μ l of 10% trichloroacetic acid (w/v) to remove proteins (centrifugation at 10,000 g, 5 min at 4°C) and to be subsequently used for glutathione determination. The rest of the supernatant was immediately frozen at –80°C pending biochemical determinations. Total proteins were quantified by the Bradford method using bovine serum albumin as a standard [24]. The left testis and the smaller liver lobe were fixed in Davidson's solution for 24 h, then washed and stored in 70% ethanol until histopathological analysis.

The remaining six individuals of each treatment were subjected to a locomotor performance test and a predatory behavioral experiment, and finally released at their collection site after a 30-d recovery period, during which they were fed noncontaminated mealworms.

Chlorpyrifos analyses

A set of solutions prepared identically to the test solutions was sent for analyses of chlorpyrifos by liquid chromatography-tandem mass spectrometry using the QuEChERS (quick, easy, cheap, effective, rugged, and safe) method [25]. The assayed concentrations of these solutions were below the nominal values anticipated: 53.4 \pm 34.3 mg/L (low dose) and 699.0 \pm 129.1 mg/L (high dose). Because the mixtures form emulsions, high variability was expected.

To determine the mean dose received by each lizard, residues of chlorpyrifos in a subsample of mealworms injected with Ciclone 48 EC were also analyzed by the QuEChERS method [25]. Mealworms were weighed and homogenized (1:10, w/v) in 0.1 M sodium phosphate buffer (pH 7.4) using a glass-Teflon Potter-Elvehjem homogenizer. Afterward, chlorpyrifos was extracted by mixing 1 g of homogenate with 1 ml of acetonitrile, high-performance liquid chromatography (HPLC)-grade. The mixture was energetically shaken by hand for 1 min. Next, 0.5 g NaCl was added and the mixture was shaken again for 30 s. Samples were centrifuged at 3,000 g for 5 min at 4°C, and a 10-ml aliquot of the supernatant was injected in an Agilent 1200 HPLC system, which included a manual injector (7725i injection valve, 20-ml loop), a vacuum degasser, a quaternary pump, and a multiple wavelength detector. The mobile phase consisted of H₂O with 0.05% acetic acid (A) and acetonitrile with 0.05% acetic acid (B). Chlorpyrifos was separated in an LC-8 column (0.46 cm \times 25 cm \times 5 mm particle size) at a flow rate of 0.5 ml/min under the following solvent program: 65% B at 0 min, increase to 95% B at 7 min, and keep for 3 min, then 65% B at 5 min. The retention time of chlorpyrifos under these chromatographic conditions was 9.14 \pm 0.024 min. The multiple wavelength detector was set at 290 nm.

Biochemical biomarkers

Antioxidant enzymes were determined on brain, intestine, liver, and testis. Glutathione S-transferase (GST; EC 2.5.1.18) activity was determined spectrophotometrically at 340 nm according to Habig et al. [26]. Specific activity was expressed as milliunits per milligram of protein using a millimolar extinction coefficient of 9.6/mM/cm. Glutathione reductase (GR; EC 1.6.4.2) activity was measured following the method described by Ramos-Martinez et al. [27]. The decrease in

absorbance at 340 nm due to nicotinamide adenine dinucleotide phosphate-oxidase (NADPH; 98%, extra pure; Sigma-Aldrich) oxidation by reduced glutathione was measured for 1 min, and a millimolar extinction coefficient of $-6.22/\text{mM}/\text{cm}$ was used for specific activity calculations. All kinetics were carried out at room temperature ($20\text{--}22^\circ\text{C}$) with blanks (reaction mixture free of sample) periodically checked for nonenzymatic reaction, and enzyme activity was corrected.

Concentrations of reduced and oxidized glutathione were fluorimetrically determined according to the method of Hissin and Hilf [28]. Reduced glutathione was determined by incubation of deproteinized samples in the presence of 1 mg/ml *ortho*-phthalaldehyde (purum, >97% HPLC-grade; Sigma-Aldrich) in Na-phosphate (0.1 M)-EDTA (5 mM) buffer (pH 8.0). Determination of oxidized glutathione concentrations required a previous step with *N*-ethylmaleimide (ultra, >99% HPLC-grade; Sigma-Aldrich) to prevent glutathione oxidation; 1 N NaOH was used instead of the phosphate-EDTA buffer. In both methods, the reaction mixture was incubated for 15 min at 20 to 22°C , with fluorescence read at 420 nm emission and 350 nm excitation. Quantification was performed using a set of external standards of reduced (3.27–327 nmol/ml) and oxidized (1.62–82 nmol/ml) glutathione, which were prepared in the same way as the samples.

Lipid peroxidation was also included as a measure of oxidative damage. The method described by Ohkawa et al. [29] was used to estimate lipid peroxidation. We used an external calibration curve made with 1,1,3,3-tetramethoxypropane (TMP; 99% purity; Sigma-Aldrich) to express lipid peroxidation as nanomoles of malondialdehyde per milligram of protein.

We measured carboxylesterase (CbE) activity in the intestine, liver, serum, and testis using two substrates: α -naphthyl acetate (α -NA; >98% purity; Sigma-Aldrich) and 4-nitrophenyl valerate (4-NPV; >98% purity; Sigma-Aldrich). Hydrolysis of α -NA was performed following the method of Gomori and Chessick [30] as adapted by Bunyan et al. [31], by which the formation of α -naphthol occurs in a reaction medium that contains 25 mM Tris-HCl (pH 7.6), 2 mM α -NA, and the sample. The hydrolytic reaction was stopped after 10 min by the addition of 2.5% (w/v) sodium dodecyl sulfate and subsequently 0.1% Fast Red ITR in 2.5% Triton X-100. Solutions were allowed to stand for 30 min in the dark, and the absorbance of the naphthol-fast red ITR complex was read at 530 nm ($\epsilon = 33.225 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$). Hydrolysis of 4-NPV by CbE activity was measured according to the method of Carr and Chambers [32]. The reaction mixture contained 1 mM 4-NPV, 50 mM Tris-HCl (pH 7.5), and the sample. The reaction was stopped after 15 min using a solution of 2% (w/v) sodium dodecyl sulfate and 2% (w/v) Tris base. The liberated 4-nitrophenolate was read at 405 nm and quantified by a calibration curve (5–100 μM). Cholinesterase activity was determined using two substrates: acetylthiocholine iodide (AcSCh; >99% purity; Sigma-Aldrich) and *S*-butyrylthiocholine iodide (BuSCh; >99% purity; Sigma-Aldrich) as described by Ellman et al. [33]. Specific ChE activity was expressed as milliunits per milligram of protein using a molar extinction coefficient of $14.15 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$. When not indicated, chemicals were purchased from Scharlab.

Zymographic method

Zymographs of esterases were carried out on nondenaturing polyacrylamide gel electrophoresis (native-PAGE) using a Bio-Rad Tetra Cell Electrophoresis Unit. Samples (10 μl) were

loaded onto 4% stacking and 12.5% resolving 0.75 mm polyacrylamide gels and electrophoresed (25 mM Tris, 192 mM glycine as running buffer) at a constant voltage of 30 V for 30 min and then 150 V. Protein bands corresponding to CbEs were visualized by incubation of the gel with a staining solution containing 100 mM Na-phosphate buffer (pH 6.5), 0.5 mg/ml α -NA, and 0.025 g of Fast Blue RR salt. The staining solution was prepared and filtered immediately before use. Gels were scanned and bands individualized in a Gel Doc EZ Imager system (Bio-Rad) and Image Lab software (Version 3.0.1, Bio-Rad).

Histopathological analyses

Fixed samples were embedded in paraffin, and 2- μm sections were cut on a rotary microtome (Leica RM 2035). Tissues were stained with hematoxylin and eosin and examined under a light microscope (Olympus BX51) using an attached Olympus camera. Liver sections were also stained with Masson's trichrome to assess liver fibrosis, with periodic acid-Schiff examined for lipid or sugar accumulation, and with Perls' Prussian Blue to verify iron pigmentation. One liver section per individual, which compromised at least 20 fields at $400\times$ was scanned, and the incidence of histopathological changes was classified through a semiquantitative scoring system: 0 = normal tissue, 1 = changes in <50% of the section, and 2 = changes in >50% of the section.

In each individual testis section, spermatogenesis development was assessed in 20 impartially chosen seminiferous tubules. Several morphological parameters were measured using image analysis software (Image J, U.S. National Institutes of Health). Tubular diameter and, along this same axis, the width of the Sertoli cell layer and the width of each spermatogenesis phase (spermatogonia, spermatocytes I and II, spermatozoa) were recorded.

Behavioral performance

Locomotor performance was evaluated by measuring maximal sprint speed on a horizontal cork substrate (2×0.1 m sprint track). Each lizard was fasted for 48 h before the start of the experiment, weighed, warmed to its optimal temperature of 33°C (M.J. Amaral, CIBIO and University of Porto, Portugal, unpublished data), and raced three times, with at least a 1-h rest interval between trials. Lizards were hand-chased through the track, and trials were recorded with a video camera (Sony/DCR-HC46, 25 fps). Following Holem et al. [34], mean maximum speed was calculated as the average of the fastest 0.20-m interval in each of the three replicate sprints and maximum speed as the fastest 0.20-m interval. Trials were repeated when lizards did not run continuously.

Predatory behavior was assessed in fasted (96 h) individuals to stimulate prey attack. Each individual was placed in an empty glass terrarium ($40 \times 20 \times 25$ cm) at optimal temperature 15 min before the start of the experiment for acclimation purposes. Test terrariums were visually isolated. At commencement of the trial, a mealworm (1.5 cm mean length) was introduced. The behavior of each lizard was recorded with a camera (Sony/DCR-HC46, 25 fps) placed on top of the terrarium. The trial was terminated if the mealworm was not consumed within 15 min. Videos were analyzed with video frame capture software (FrameShots, EOF Productions), recording the time each individual took to attack the mealworm (time of latency to attack) and the time each took to subdue and swallow the prey (manipulation time).

Statistical analyses

The results obtained for each biomarker from the treatment groups were compared to the control using analyses of variance (ANOVA), analyses of covariance (ANCOVA), multiple analyses of covariance (MANCOVA), and/or repeated measures analysis of variance followed by a Dunnett's multiple comparison post hoc test. Treatment was the dependent variable, and snout-vent length and body mass were used as covariates to check significant interactions. When the assumptions for analysis of variance were not met (i.e., homogeneity of variance, normality), we used the nonparametric Kruskal-Wallis test to compare data. Behavioral data were ranked prior to the analyses. Differences in the prevalence and intensity of histological changes in liver between treatments were compared with the control using Pearson's chi-squared test. Level of significance was $\alpha = 0.05$.

RESULTS

During the entire period of the experiment, animals were exposed on each feeding day to 0.53 ± 0.34 and 6.99 ± 1.29 μg of chlorpyrifos per lizard in the low- and high-dose groups, respectively (data not shown). This is equivalent to a mean chlorpyrifos exposure of 0.12 (0.05–0.17) mg/kg/d in the low-dose group and 1.57 (1.46–1.65) mg/kg/d in the high-dose group every other day during a period of 20 d. The dosages in mealworms were within the expected values of 2.38 μg of chlorpyrifos/g of mealworm in the low-dose group and 23.68 μg of chlorpyrifos/g of mealworm in the high-dose group (1 g of mealworm corresponds to 10–12 individuals). No animals died or showed any visible symptoms of cholinergic poisoning during the exposure period.

Biochemical biomarkers

In general, exposure to chlorpyrifos did not cause any significant variation in the activity of the antioxidant enzymes (GST and GR), lipid peroxidation, or glutathione concentrations (reduced glutathione [GSH] and oxidized glutathione [GSSG]) in the different tissues examined (Table 1). The results differed on a tissue basis, and in general, there was high individual variability. For example, GST activity was highest in liver, followed by testes, intestine, and brain. Whereas GR activity was more stable between treatments and had similar levels in

intestine, liver, and testis, and was 10 times lower in brain. Lipid peroxidation was higher in liver and intestine than in testis and showed a slight increase with exposure in intestine and testis. The only significant difference we detected in the oxidative stress biomarkers was in GSH concentrations in brain after exposure to the low dose of chlorpyrifos (ANOVA treatment, $F_{2,14} = 5.2$, $p = 0.02$). Higher concentrations of GSH also occurred in intestine, and there was a slight increase in GSH content in testes with chlorpyrifos exposure; but in all cases there was high individual variability, and GSSG levels remained similar between treatments. In both brain and intestine, the concentrations of GSH and GSSG were similar and the ratio of GSH to GSSG was close to one. Total glutathione content was higher in liver when compared with other tissues, whereas the ratio of GSH to GSSG was higher in testes.

Activity of CbE was recorded in all tissues and decreased in a dose-dependent manner with exposure to chlorpyrifos (Figs. 1 and 2a). Esterase activity was tissue (intestine, liver, serum, testis) and substrate (α -NA and 4-NPV) specific. Statistically significant differences in enzyme activities were detected in all tissues except testis. Significant differences in CbE activity using α -NA (Fig. 1a) were detected in the two treatment groups when intestine (ANOVA treatment, $F_{2,14} = 22.9$, $p < 0.001$) and liver (ANOVA treatment, $F_{2,14} = 45.7$, $p < 0.001$) were used as esterase sources. In serum (Fig. 2a), CbE activity was inhibited in lizards exposed to the higher dose (Kruskal-Wallis treatment, $H_{2,17} = 11.1$, $p = 0.004$). With the α -NA substrate, CbE activities were inhibited by 56/85%, 50/81%, and 47/51% (low dose/high dose) in intestine, liver, and testis, respectively. In serum, a slight induction was found in the low-dose lizards, 112%, whereas an 86% inhibition was found in the high-dose group. The highest activity using α -NA was found in serum, followed by intestine. Similarly, CbE activity with the 4-NPV substrate (Figs. 1b and 2a) was also significantly inhibited in both treatment groups in liver (ANOVA treatment, $F_{2,14} = 12.6$, $p < 0.001$). In intestine and serum, inhibition was significant depressed only in the high-dose group (intestine ANOVA treatment, $F_{2,14} = 9.1$, $p = 0.003$; serum Kruskal-Wallis treatment, $H_{2,17} = 11.1$, $p = 0.004$). Levels of CbE inhibition with 4-NPV substrate were 55/87%, 57/80%, and 34/54% in intestine, liver, and testis, respectively. The highest CbE activities with 4-NPV were found in serum, followed by liver. In general, CbE activities were twice as high with the 4-NPV substrate.

Table 1. Oxidative stress parameters in *Podarcis bocagei* after a 20-d exposure to chlorpyrifos^a

Biomarker	n	GST	GR	GSH	GSSG	tGS	GSH/GSSG	LPO	
		(mU/mg protein)	(mU/mg protein)	(ng/mg protein)	(ng/mg protein)	(ng/mg protein)		(ng MDA/mg protein)	
Brain	Control	6	31.5 ± 3.2	5.0 ± 0.8	17.3 ± 3.5 ^b	23.0 ± 13.3	40.2 ± 16.1	1.1 ± 0.8	
	Low dose	5	78.9 ± 86.8	5.4 ± 1.5	22.0 ± 12.7 ^b	29.9 ± 28.0	51.9 ± 39.1	1.2 ± 1.3	
	High dose	6	44.7 ± 14.5	4.84 ± 2.4	18.3 ± 4.8	15.0 ± 12.8	33.2 ± 15.6	1.9 ± 1.4	
Intestine	Control	6	374.2 ± 137.0	77.8 ± 18.1	18.8 ± 6.6	21.5 ± 7.9	40.2 ± 10.1	1.0 ± 0.5	4,511.3 ± 2,669.3
	Low dose	6	268.4 ± 130.8	78.2 ± 35.5	23.4 ± 15.1	23.2 ± 5.3	46.7 ± 16.4	1.0 ± 0.6	6,132.8 ± 3,122.1
	High dose	5	358.0 ± 271.2	75.2 ± 16.7	12.0 ± 5.1	18.8 ± 4.3	30.8 ± 7.8	0.6 ± 0.3	7,475.8 ± 2,298.3
Liver	Control	6	1,428.0 ± 290.7	63.4 ± 12.2	81.7 ± 14.6	39.9 ± 23.5	121.6 ± 28.8	2.8 ± 1.9	9,457.7 ± 2,633.2
	Low dose	5	1,748.7 ± 553.3	83.3 ± 49.0	61.3 ± 40.2	39.8 ± 14.0	101.1 ± 35.5	1.8 ± 1.4	12,481.5 ± 6,905.0
	High dose	6	1,277.0 ± 396.7	61.6 ± 7.2	64.8 ± 27.6	26.8 ± 5.3	91.6 ± 25.2	2.6 ± 1.2	8,809 ± 1,821.8
Testis	Control	6	607.4 ± 352.9	49.0 ± 30.5	41.9 ± 22.8	10.9 ± 9.0	57.8 ± 21.3	5.4 ± 3.2	8,668.8 ± 2,008.4
	Low dose	6	567.2 ± 119.0	53.5 ± 34.1	58.8 ± 35.8	8.8 ± 4.8	67.7 ± 39.5	6.7 ± 4.2	12,237.5 ± 8,899.4
	High dose	6	546.6 ± 137.6	53.5 ± 29.4	66.6 ± 34.5	10.3 ± 4.7	76.9 ± 34.7	7.3 ± 4.6	12,812.1 ± 3,439.4

^a Control (0 mg/kg/d body wt), low dose (0.12 mg/kg/d body wt), and high dose (1.57 mg/kg/d body wt). All parameters are presented as means ± standard deviations.

^b Data that are significantly different from controls following Dunnett's post hoc results after analysis of variance (ANOVA) or post hoc paired comparisons after Kruskal-Wallis, $p < 0.05$.

GST = glutathione S-transferase; GR = glutathione reductase; GSH = reduced glutathione; GSSG = oxidized glutathione; tGS = total glutathione; LPO = lipid peroxidation; MDA = malondialdehyde.

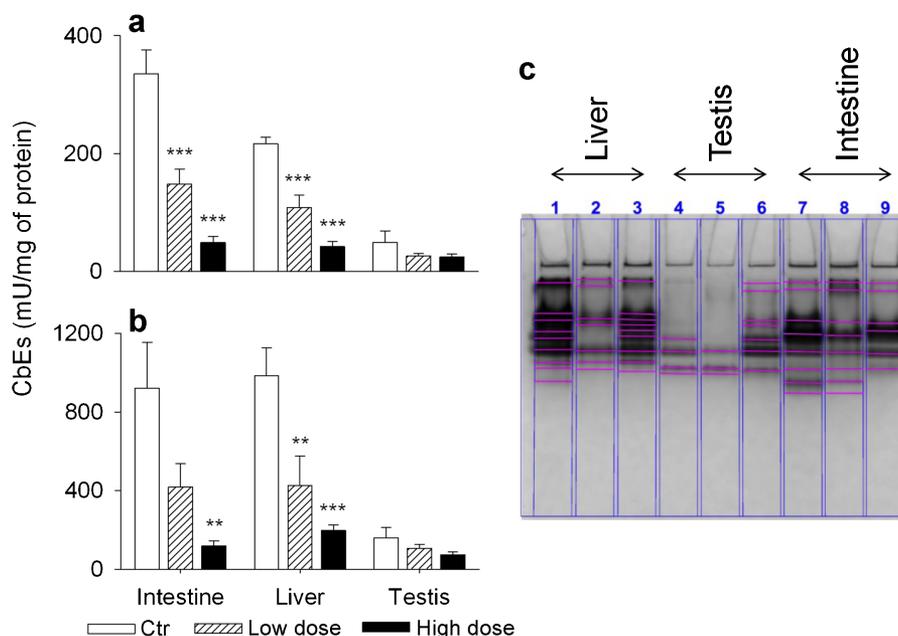


Fig. 1. Carboxylesterase (CbE) activity in intestine, liver, and testis in *Podarcis bocagei* exposed to sublethal concentrations of chlorpyrifos for a period of 20 d. Control (Ctr) (0 mg/kg/d body wt), low dose (0.12 mg/kg/d body wt), and high dose (1.57 mg/kg/d body wt). Esterase activity was measured using the substrates (a) α -naphthyl acetate (α -NA) and (b) 4-nitrophenyl acetate (4-NPV). $n = 6$, except in low dose (liver and serum) and high dose (intestine), where $n = 5$. Asterisks are Dunnett's post hoc results after analysis of variance (ANOVA) or post hoc paired comparisons after Kruskal-Wallis: ** $p < 0.01$, *** $p < 0.001$. (c) Zymograph of CbE activity in intestine, testis, intestine where 1, 4, and 7 are control; 2, 5, and 8 are high-dose; and 3, 6, and 9 are low-dose. Data are expressed as mean \pm standard error. [Color figure can be seen in the online version of this article, available at [wileyonlinelibrary.com](http://www.interscience.wiley.com)]

We distinguished multiple CbE isoforms by native PAGE, which was tissue-specific (Fig. 1c). Liver had the higher abundance of CbE isoforms (nine protein bands). Furthermore, the relative mobility of most of the stained bands was similar among tissues, which suggests the occurrence of the same proteins displaying CbE activity in the selected tissues. The staining intensity of CbE bands decreased in lizards exposed to chlorpyrifos irrespective of the tissue.

The use of two selective substrates allowed us to distinguish different ChEs in the analyzed tissues. Marked variations were noted on a tissue basis, and ChE activity was significantly inhibited in the high-dose group in all tissues (Figs. 2b and 3). In brain, ChE activity was slightly induced in the

low-dose group, 123% of control activity, and inhibited by 70% in the high-dose group (ANOVA treatment, $F_{2,14} = 16.2$, $p < 0.001$). Intestinal ChE activity was induced by 149% and significantly inhibited by 82% in the low- and high-dose groups, respectively (ANOVA treatment, $F_{2,13} = 48.2$, $p < 0.001$). Similarly, serum ChE activity toward AcSCh was induced by 112% and significantly inhibited by 82% in the low- and high-dose groups (ANOVA treatment, $F_{2,14} = 26.6$, $p < 0.001$), respectively. However, hydrolysis of the substrate BuSCh by serum ChE activity was significantly inhibited in both treatments, by 57 and 96%, respectively (ANOVA treatment, $F_{2,13} = 52.7$, $p < 0.001$).

Histopathological analyses

Livers from control individuals were characterized by normal tissue with some signs of congestion (three out of six

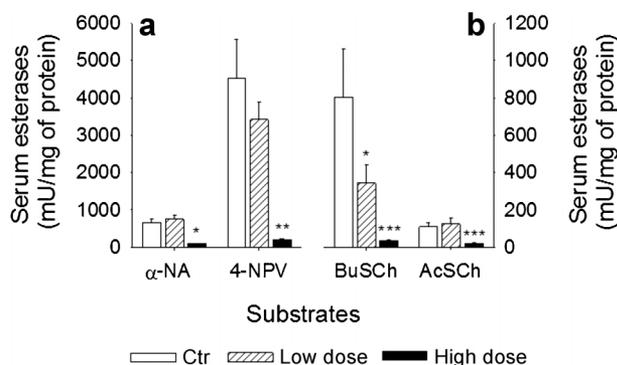


Fig. 2. Esterase activity in serum in *Podarcis bocagei* exposed to sublethal concentrations of chlorpyrifos for a period of 20 d. Control (Ctr) (0 mg/kg/d body wt), low dose (0.12 mg/kg/d body wt), and high dose (1.57 mg/kg/d body wt). Esterase activity was measured using the substrates α -naphthyl acetate (α -NA), 4-nitrophenyl acetate (4-NPV), acetylthiocholine iodide (AcSCh), and *S*-butyrylthiocholine iodide (BuSCh). $n = 6$, except in low dose ($n = 5$). Asterisks indicate statistically significant results of Dunnett's post hoc tests after analysis of variance (ANOVA) or post hoc paired comparisons after Kruskal-Wallis: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data are expressed as mean \pm standard error.

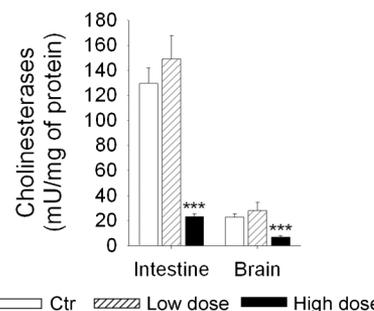


Fig. 3. Cholinesterase activity in brain and intestine in *Podarcis bocagei* exposed to sublethal concentrations of chlorpyrifos for a period of 20 d. Control (Ctr) (0 mg/kg/d body wt), low dose (0.12 mg/kg/d body wt), and high dose (1.57 mg/kg/d body wt). Cholinesterase activity was measured in brain using the substrate acetylthiocholine iodide and in intestine using *S*-butyrylthiocholine iodide. $n = 6$, except in intestine-high dose ($n = 5$). Asterisks represent significant differences following Dunnett's post hoc tests: *** $p < 0.001$. Data are expressed as mean \pm standard error.

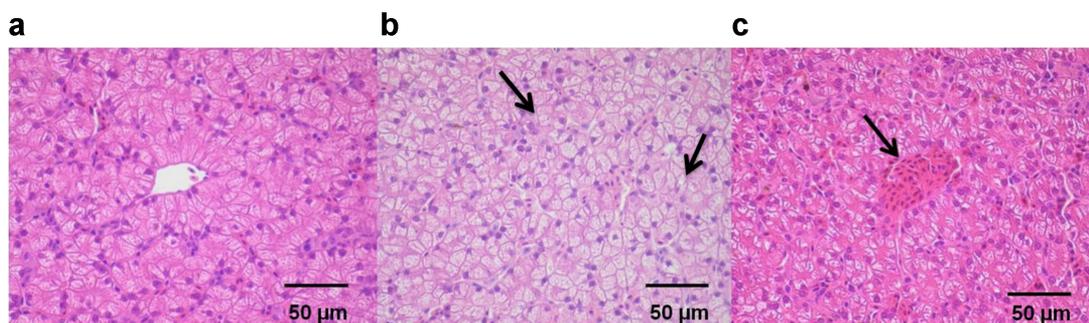


Fig. 4. Representative liver sections of *Podarcis bocagei* exposed to sublethal concentrations of chlorpyrifos for a period of 20 d. (a) Liver section of a control animal representing a normal liver (hematoxylin and eosin 400 \times). (b and c) Examples of liver sections of individuals exposed to the high dose (699.0 ± 129.1 mg/L). (b) Liver section with hepatocyte vacuolation (arrows, hematoxylin and eosin 400 \times). (c) Liver section with congestion (arrow, hematoxylin and eosin 400 \times). [Color figure can be seen in the online version of this article, available at [wileyonlinelibrary.com](http://www.wileyonlinelibrary.com)]]

individuals, Fig. 4a). One of these individuals presented hepatocyte degeneration, vacuolation, and fibrosis. Livers of individuals from both treatment groups consistently presented congestion and hepatocyte vacuolation. In general, lizards in the high-dose group presented a higher prevalence of histological changes, including severe congestion, hepatocyte vacuolation, and light fibrosis (Fig. 4b and c). In the high-dose treatment, the incidence of fibrosis, hepatocyte degeneration, and vacuolation approached significance when compared with the control (Pearson chi-squared tests, $df=2$: fibrosis $\chi^2=5.1$, $p=0.08$; congestion $\chi^2=9.1$, $p=0.01$; hepatocyte degeneration $\chi^2=4.4$, $p=0.11$; Pearson chi-squared tests, $df=4$: hepatocyte vacuolization $\chi^2=6.6$, $p=0.15$).

Testes histology presented no alterations in morphology, irrespective of exposure dose (data not shown). In both control and treated groups, normal testes with full spermatogenic activity were observed. Seminiferous tubule diameter was not different in any treatment group when compared to control animals (ANCOVA treatment, $F_{2,14}=0.04$, $p=0.96$). Additionally, no size differences were detected for the Sertoli cell layer (Kruskal-Wallis treatment, $H_{2,18}=1.7$, $p=0.42$) or for the layers of the different phases of spermatogenesis: spermatogonias (ANCOVA treatment, $F_{2,14}=0.4$, $p=0.70$), spermatocyte I (ANCOVA treatment, $F_{2,14}=0.05$, $p=1.0$), spermatocyte II (ANCOVA treatment, $F_{2,14}=0.2$, $p=0.84$), and spermatozoa (ANCOVA treatment, $F_{2,14}=0.1$, $p=0.90$).

Behavioral performance

Mean maximum speed and maximum speed were not affected by exposure to chlorpyrifos (ANCOVA treatment mean maximum speed, $F_{2,14}=1.7$, $p=0.22$; ANCOVA treatment maximum speed, $F_{2,14}=0.5$, $p=0.64$). In the predatory behavior experiment, only one of the 18 lizards did not consume the prey item in the 15-min period. Considering time of latency to attack, an outlier was removed from the data (value $> \text{mean} \pm 2$ SD), and no difference between exposures was found (ANCOVA treatment time of latency to attack, $F_{2,12}=1.5$, $p=0.27$). For manipulation time our results, although not statistically significant, suggest that animals exposed to chlorpyrifos take more time to manipulate the prey (ANCOVA treatment manipulation time, $F_{2,13}=3.1$, $p=0.08$). Considering both variables together, lizards in the high-dose treatment took significantly more time to subdue and swallow the prey than lizards from the control group (Fig. 5) (MANCOVA treatment, Wilks $F_{4,22}=2.8$, $p=0.048$).

DISCUSSION

Reptiles inhabiting agroecosystems can be exposed to pesticides through the ingestion of contaminated food. However, detection of pesticide exposure in the field is usually complex because there are always multiple and fluctuating biological and environmental variables that impact pesticide uptake and sublethal effects. This limitation of using field data is compounded when the understanding of the biochemistry and physiology of the organism of interest (e.g., lizards) is limited. When exposure results in toxic effects, they are usually sublethal in nature; and thus, their occurrence under natural conditions is difficult to resolve because they can be masked by other nontoxicological variables. Laboratory studies sacrifice ecological realism but allow a higher degree of control over exposure parameters, allowing detection of those sublethal effects. Our results suggest that sublethal, subchronic exposure to chlorpyrifos can affect *P. bocagei* in a dose-dependent manner. Adverse effects of ecologically realistic chlorpyrifos doses occurred at both the subindividual and individual levels, including differences in esterase activities, liver histopathological changes, and altered predatory behaviors.

Glutathione-dependent enzymes, which are involved in cellular antioxidant defense, have been studied in reptiles, mainly to assess the response to overwintering and hypoxic conditions (reviewed in Mitchelmore et al. [35]). Induction of glutathione peroxidase after exposure to cadmium was reported for adult male *Uromastix aegyptius* lizards [36]. Nevertheless, glutathione-dependent enzymes have the potential to limit the damage caused by reactive oxygen species generated during

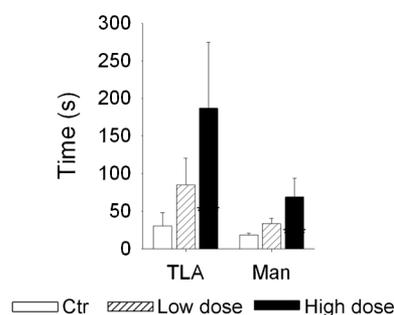


Fig. 5. Predatory behavior, assessed by time of latency to attack (TLA) and manipulation time (Man), in *Podarcis bocagei* exposed to sublethal concentrations of chlorpyrifos for a period of 20 d. Ctr (0 mg/kg/d body wt), low dose (0.12 mg/kg/d body wt), and high dose (1.57 mg/kg/d body wt). Bars represent means + SE ($n=6$, except for low dose $n=5$).

pesticide-detoxification processes and have been used in several studies to assess pesticide exposure and effects (reviewed for fish in van der Oost et al. [37]). Lipid peroxidation has been used as a quantitative measure of the extent of oxidative damage caused by contaminants. In the present study, we found no signs of oxidative stress caused by chlorpyrifos exposure. This is contrary to the results of a study in rats, for which a dose of 6.75 mg/kg body weight for a period of 28 d resulted in a significant increase in serum lipid peroxidation and decreased significantly the activity of serum GST [38]. Another rat study also reported accumulation of malondialdehyde in different tissues and a dose-dependent decrease in antioxidant enzymes after 1 d of exposure to 38.8 mg/kg body weight [3]. In both studies, rats were orally exposed to a higher dosage of chlorpyrifos than the higher one in the present study.

Chlorpyrifos exposure resulted in the inhibition of CbEs in all tissues analyzed in a dose-dependent manner, except for serum CbE, which was strongly depressed at the higher dose of the OP. Inhibition of CbE activity has been used as a biomarker of OP exposure in different species, and several authors have suggested that CbEs can have a protective role by binding stoichiometrically to OP pesticides, decreasing OP concentration and toxicity [39,40]. Thus, effects of pesticide exposure can be alleviated in different tissues by the levels of CbE activity, its sensitivity to chlorpyrifos inhibition, and the presence of multiple isozymes [39]. Different studies have also suggested that CbE activity could be a more sensitive biomarker than brain cholinesterase activity, the most commonly used biomarker of OP exposure [41]. It is widely accepted that CbE activity provides a protective effect against OP exposure because of its higher sensitivity to inhibition by these agrochemicals compared to ChE activity [41]. Results in the present study support the aforementioned effect. Animals exposed to the lower dose of chlorpyrifos showed significant inhibition of CbE activity in the liver and intestine, whereas this depression was not found in the brain and intestine ChE activities (Figs. 1 and 3). Sanchez-Hernandez and Wheelock [40] suggested that intestinal CbEs would have an important role in OP detoxification as one of the first surfaces of contact for feeding exposure. These results reinforce the need to study different tissues and substrates as different isozymes seem to be involved in the hydrolysis of the different substrates in multiple tissues. The only tissue where no significant differences were found for CbE activity was the testis, although a general trend of activity depression with increased dose was observed. Several studies with mammals and other organisms have demonstrated that CbEs have an important role in reproduction by altering the metabolic routes of testosterone production [5]. In the present study, enzymatic activity was dependent on tissue and substrate. The protective role of CbEs was not clear in serum CbE activity, but it was apparent in both liver and intestine CbE activities. Liver CbEs were strongly inhibited after exposure to both doses of chlorpyrifos. Similarly, intestinal CbE activity was also depressed. However, we detected higher intestinal CbE activity in control than in liver with the α -Na substrate.

Cholinesterases, in particular AChE, have been intensively studied as biomarkers of pesticide exposure and effect, in particular for OP and carbamate pesticides. In reptile brains, AChE is expected to represent >90% of total ChEs (S.R. Schmidt, 2003, Master's thesis, Texas Tech University, Lubbock, TX, USA). The relationship between AChE inhibition and death has been intensively debated. Reductions of 50 to 80% in AChE activity have been related to mortality in different vertebrate species (e.g., in birds [42]). In the present study,

inhibition levels of 70% of brain ChE activity recorded in the group exposed to the high dose did not result in the death of any individual. These levels of inhibition are similar to those described for the lizard *Gallotia gallotia* after acute exposure to parathion, where ChE inactivation levels of 84 and 70% were observed without mortality [43].

Even if not lethal, chlorpyrifos can interfere with brain ChEs and the activity of the central nervous system, impairing essential behavioral functions, like predator-prey interactions [44]. Cholinesterases in other tissues, such as blood, have been suggested to have, like CbEs, a protective role over brain ChE, detoxifying OPs before they reach the brain [41]. Different studies in lizards have further compared the levels of ChE in those tissues (mainly serum) and found them to be correlated with those of brain ChE [43]. In the present study, this relationship could only be established with serum ChE (assessed with BuSCh substrate). In reptiles, 80% of the total ChE activity in serum is attributable to BChE activity [11,13]. Serum ChE (BuSCh substrate) was inhibited at the low dose before any effect was observed in brain ChE. Cholinesterase in intestine and serum (AcSCh substrate) was not inhibited at the low dose. At the high dose, ChEs were strongly inhibited in all tissues, so any protective role would be lost. Sanchez-Hernandez et al. [43] obtained similar results to the findings presented here, which show an exponential decay of AChE activity when serum BChE reached 90% of inhibition.

We detected a higher prevalence of liver histopathological changes in animals exposed to chlorpyrifos. These changes occurred mainly in the high-dose group and included fibrosis, hepatocyte degeneration, and vacuolation. Despite being statistically nonsignificant, these alterations might be indicative of metabolic stress. Studies in rats have demonstrated a link between exposure to chlorpyrifos and the appearance of similar lesions [38,45]. Toxic effects of chlorpyrifos on testicular function have also been noted in rats [5,9]. Nevertheless, in the present study, no effects were noted. Normal testicular histology with all the successive stages of spermatogenesis was observed in the three groups.

If exposure to pesticides results in behavioral alterations, growth, reproductive success, and survival might be affected. Locomotor performance is probably the individual parameter that has been employed most in studies with reptiles exposed to insecticides. Nevertheless, these studies seem to indicate that this flight response is not a parameter sufficiently sensitive to address pesticide exposure [46,47]. In the present study, the results similarly show this test to be an insensitive biomarker of chlorpyrifos exposure, with no difference between control and treated animals. In contrast, predatory behaviors provide a more sensitive indicator of locomotor impairment because they involve multiple neuromuscular systems, rather than simply a sympathetically controlled, reflexive flight response. Bain et al. [13] observed that animals exposed to a high dose of fenitrothion tended to make more attempts to catch prey. The authors speculated that this type of response could be related to decreased visual acuity, changes in muscle activity, or decreased muscle coordination [13]. We found that after exposure to chlorpyrifos, animals took more time to capture and swallow a prey item, despite prior habituation to the prey. Inhibition of ChEs, which was demonstrated in the same animals, provides a mechanistic explanation. Inhibition of AChE causes accumulation of acetylcholine in synaptic junctions. The accumulation of acetylcholine can alter neuromuscular abilities and, thus, decrease the muscle coordination essential for the manipulation of prey.

We can presume that animals will recover when the exposure terminates. However, if we take into consideration the slow rate of esterase recovery that has been reported for reptiles [13,43], these results can represent a high exposure risk. Animals would be impaired not only during the exposure period but also for a significant period afterward. Insecticide application usually occurs during spring and autumn, the period when animals are acquiring energy for production and reproduction and to survive the winter. To fulfill energetic requirements, compromised individuals would have to spend more time foraging to catch the same amount of prey. During foraging, lizards are potentially at a higher risk of predation. Thus, we can speculate that if an animal compensates for deficiencies in predatory ability by foraging for longer periods, it is increasing its risk of predation. On the other hand, if an animal does not compensate, then reduced feeding rates will have implications for its energetic balance.

Our exposure rates were under the median lethal concentration (LC50) values reported for several vertebrate species including birds (5–157 mg/kg body wt) and mammals (151–1,000 mg/kg body wt) [48]. For amphibians, the reported doses are given as concentration in the aquatic medium and not easily comparable, ranging from 2.4 to 66.2 µg/L, depending on the species [48]. Given the lack of data, it is difficult to compare the toxicity of chlorpyrifos to reptiles and other terrestrial vertebrates and, thus, evaluate if birds can be used in risk-assessment processes as surrogate species.

CONCLUSIONS

In the present study, we demonstrated that a subchronic exposure to environmentally relevant doses of chlorpyrifos can inhibit esterases and impair predatory behaviors. It remains to be seen if the biochemical and behavioral effects observed in the present study are likely to translate into any short- or long-term implications for populations. Further work in the form of long-term mesocosm experiments will be needed to examine these issues.

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